Aminoglycoside Antibiotics Are Able To Specifically Bind the 5′-Untranslated Region of Thymidylate Synthase Messenger RNA†

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ABSTRACT: The translational initiation codon for thymidylate synthase (TS) mRNA is located in a unique stem−loop structure which contains an internal cytosine-cytosine (CC) bubble. This stem−loop structure is thought to be important in the regulation of TS translation, which is itself an important target for anticancer drugs, such as 5-fluorouracil. Internal bubble or bulge structures are candidate receptors for the aminoglycoside antibiotics. It is shown here that aminoglycosides bind in a specific and saturable fashion with dissociation constants of approximately 1 μM to a TS mRNA site 1 construct and that the binding site for the aminoglycosides is located in the CC bubble region. In fact, the CC bubble, when grafted into other stem−loop structures, confers aminoglycoside binding on them. These studies reveal an additional binding domain for aminoglycosides and also suggest how novel anti-cancer drugs might be designed that affect TS mRNA translation rather than enzyme function.

Many antibiotics, including the aminoglycosides and tetracyclines, operate pharmacologically by binding to functionally important regions of RNA (*1*−*6*). While the 1,3-(2)-amino alcohol moieties in these molecules appears to be of central importance in allowing them to bind to their RNA targets (*7, 8*), little is known about the local structure of RNA molecules that allows high affinity and specific binding of these drugs. In studies on RNA molecules that are either selected to bind to particular aminoglycosides (*9*−*11*) or are naturally occurring target RNA molecules for aminoglycosides (*12*−*17*), it appears that internal bulges or bubbles are part of the preferred binding sites. Internal bulges are part of the aminoglycoside binding-site aptamers selected against the aminoglycoside tobramycin (*11*) and in human immunodeficiency virus (HIV) RRE RNA (*12, 13*), while an internal bubble structure confers aminoglycoside binding on the bacterial A-site decoding region ribosome RNA (rRNA) (*14*−*18*). Aminoglycosides, at least, do not appear to be able to bind with high affinities to either loop structures or to duplex regions of RNA. To further characterize the local domain nature of RNA structure that might support aminoglycoside binding, we have been interested in studying naturally occurring RNA molecules that might contain aminoglycoside binding domains. It is shown here that the 5′-UTR[1] (untranslated region) of the mRNA of thymidylate synthase (TS) contains an aminoglycoside binding domain that is localized to an internal CC bubble.

Thymidylate synthase (TS) catalyzes the reductive methylation of 2′-deoxyuridine 5′-monophosphate (dUMP) to form thymidine 5′-monophosphate (dTMP). TS is the sole *de novo* source of dTMP formation in cells; it is critical for DNA synthesis and is an important target enzyme in cancer chemotherapy (*19*−*23*). Interestingly, TS has been shown to bind to numerous RNA constructs (*24, 25*). Of particular interest is the fact that TS is able to bind specifically to its own mRNA with an affinity in the 1−2 nM range (*26, 27*). This binding has a functional consequence, because the translation of the human TS mRNA has been shown to be controlled by TS via an autoregulatory feedback mechanism. The binding of apo-TS to its own message prevents its translation. TS is one of the first eukaryotic enzymes demonstrated to be regulated in this fashion (*28*). This mode of translational repression appears to have a general theme in metabolic regulation in eukaryotes (*29*).

There are two distinct domains in the TS mRNA that are involved in protein recognition. (Figure 1) (*30*). The first site (site 1) is confined to a predicted hairpin loop located within the first 188 nucleotides of the message and includes the initiation codon AUG in the loop region (*30*). The second site (site 2) appears to be confined to an approximately 200 nucleotide region corresponding to nucleotides (nts) 434−634 in the protein coding region. The sequence of the site 1 hairpin loop has recently been reported (*30*), and the predicted secondary structure of the hairpin loop is shown in Figure 2. Given that the predicted structure of site 1 contains a CC bubble, it was of interest to determine whether aminoglycosides could specifically bind to a site 1 construct and, further, whether the binding-site is localized to the putative bubble region.

We have recently developed sensitive fluorescence methods that allows for direct and quantitative binding measurements of aminoglycoside−RNA interactions (*11, 31, 32*). The basis of this methodology involves the use of fluorescent aminoglycoside conjugates to bind to the RNA molecule.

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[1] Abbreviations: nts, nucleotides; K_d, dissociation constant; TS, thymidylate synthase; 5′-UTR, 5′-untranslated region; CC, cytosine-cytosine; ENU, ethyl nitrosourea; PCR, polymerase chain reaction; RRE, Rev response element.

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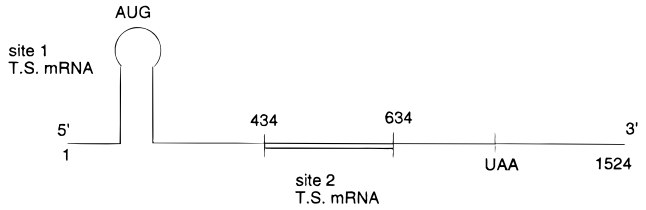
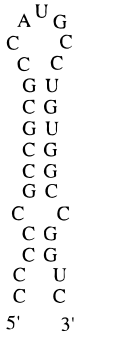


FIGURE 1: Zuker fold analysis of the two binding sites of TS mRNA (*22*), in which the site 1 TS mRNA is predicted to adopt a stable stem−loop secondary structure with the translational AUG start site contained within its hairpin loop.



w.t. site 1 TS mRNA

FIGURE 2: Predicted secondary structure of the wt site 1 TS mRNA (*30*). The translational start codon AUG is contained in the hairpin loop.

Detection is by either fluorescence polarization or quenching methods (*11, 32*). By use of this technology, it is demonstrated here that a site 1 TS mRNA construct specifically binds aminoglycosides and, further, that this binding importantly depends on the CC bubble of the stem−loop. This study provides an additional RNA domain capable of specifically binding aminoglycoside antibiotics.

MATERIALS AND METHODS

Materials

The aminoglycosides neomycin B, gentamycin C, kanamycin B, tobramycin, paramomycin, streptomycin, and hygromycin were purchased from either Sigma or Fluka. The primers and the DNA used for the synthesis of the 33-mer site 1 TS mRNA (construct **1**) were purchased from Integrated DNA Technologies. GeneAmp PCR kit is from Perkin-Elmer, and the Ribomax large-scale RNA T7 production kit was purchased from Promega. RNase CL3 was purchased from Boehringer Mannheim, ethyl nitrosourea (ENU) was purchased from Sigma, and aniline was purchased from Aldrich. *N*-hydroxysuccimide-derivatized Affi-Gel 10 was from Bio-Rad and Sephadex G-50 was from Pharmacia. 5-Carboxytetramethylrhodamine-labeled paramomycin (CRP), 5-carboxytetramethylrhodamine-labeled gentamycin (CRG), 5-carboxytetramethylrhodamine-labeled kanamycin (CRK), and 5-carboxytetramethylrhodamine-labeled tobramycin (CRT) were synthesized according to previous reports (*31*).

Methods

Preparation of RNA Constructs. The double-stranded DNA was constructed by large-scale PCR amplification with the GeneAmp PCR kit. Purification of dsDNA was performed through electrophoresis with polyacrylamide gels and purification by the crush and soak method in 0.3 M Na_2SO_4 (*33*). The RNA of the site 1 TS mRNA was then prepared by transcription of the PCR products with the Ribomax large-scale RNA T7 production kit. The RNA was then purified by gel electrophoresis with 15% denaturating polyacrylamide gels. RNA concentrations were determined spectrophotometrically at 260 nm.

*Fluorescence Measurements.*The affinities of both the rhodamine-derivatized and underivatized aminoglycosides for the site 1 TS mRNA were determined by fluorescence anisotropy. Fluorescence measurements were performed on a LS-50B spectrofluorometer (Perkin-Elmer) at 20.0 ± 0.1 °C in the incubation buffer [150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES (pH = 7.4)]. The samples were excited at 550 nm, and fluorescence was monitored at 580 nm. Slit widths on both the excitation and emission sides were 10 nm for measuring aminoglycoside−TS mRNA binding. The integration time was 5 s. For every single point, five measurements were made, and their average values were used for calculations. Equation 1 is used for the calculation of K_d (dissociation constant) by curve-fitting the fluorescence anisotropy measurements between the aminoglycosides and site 1 TS mRNA (assuming a 1:1 complex)

$$I = I_0 + 0.5\Delta\epsilon([\text{labeled aminoglycoside}]_0 - \{([\text{labeled aminoglycoside}]_0 + [\text{RNA}]_0 K_d)^2 - 4[\text{labeled aminoglycoside}]_0[\text{RNA}]_0\}^{0.5} \quad (1)$$

where I_0 and I are the fluorescence anisotropy of rhodamine-derivatized aminoglycoside in the absence and presence of site 1 TS mRNA, respectively. $\Delta\epsilon$ is the difference between the fluorescence anisotropy of 1 μM rhodamine-derivatized aminoglycoside in the presence of an infinite concentration of RNA and in its absence. $[\text{RNA}]_0$ is the total concentration of RNA added; $[\text{labeled aminoglycoside}]_0$ is the total concentration of rhodamine-derivatized aminoglycoside.

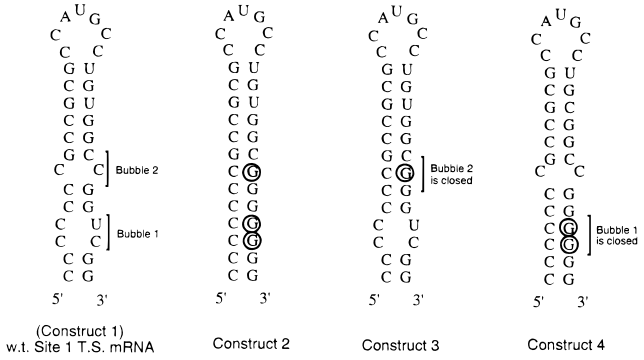
The competition binding measurements between aminoglycoside and CRP−RNA complex were performed at constant concentrations of CRP and TS mRNA by monitoring fluorescence anisotropy changes as a function of the changing concentrations of the aminoglycoside. The K_ds for the selected aminoglycoside were subsequently calculated by curve-fitting the fluorescence anisotropy measurements of CRP as a function of the aminoglycoside:

$$[\text{aminoglycoside}]_0 = (K_d(A_\infty - A)/K_d(A - A_0) + 1)\{[\text{RNA}]_0 - K_d(A - A_0)/(A\infty - A) - [\text{CRP}]_0(A - A_0)/(A_\infty - A_0)\} \quad (2)$$

where K_d is the dissociation constant between the TS mRNA and the aminoglycoside, $[\text{aminoglycoside}]_0$ is the initial concentration of the aminoglycoside, and A, A_∞, and A_0 are the fluorescence anisotropy values of sample, totally bound labeled aminoglycoside, and totally free labeled aminoglycoside, respectively.

Ethylation Modification Interference Assay. 5′-^{32}P-Labeled site 1 TS RNA (2×10^6 cpm) was modified with ethyl nitrosourea (ENU) under denaturing conditions as described (*34*). After modification, the RNA was precipitated with

Chart 1: A Series of Mutagenized Site 1 TS mRNA Constructs



ethanol and lyophilized. Modified RNAs were loaded onto a neomycin column and eluted with a salt gradient and several fractions were collected. To cleave RNAs at the modified phosphate, RNAs were resuspended in 20 μL of 100 mM triethylammonium bicarbonate, pH 9.0, and heated at 50 °C for 5 min. RNAs were then lyophilized and analyzed on 15% polyacrylamide/8 M urea gels.

RNase CL3 Footprinting Assay of TS RNA. 5′-^{32}P-Labeled TS RNA (20 kcpm) in 40 μL of incubation buffer [150 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 20 mM HEPES (pH = 7.4)] was treated with RNase CL3 in the presence or absence of neomycin for 4 min at 25 °C. The reaction was stopped by addition of a 3 M sodium acetate solution containing 25 mM EDTA and then the RNA was precipitated with ethanol. Electrophoresis was carried out for 2 h on 15% polyacrylamide/8 M urea gels.

RESULTS

Binding Characteristics of Rhodamine-Derivatized Aminoglycosides to a Site 1 TS mRNA Construct. The site 1 TS construct, whose Mfold (*35*) minimized structure is shown in Chart 1 (construct **1**), was prepared and studied with respect to its ability to specifically bind aminoglycosides. The construct prepared contains the site 1 TS mRNA domain plus two extra GC base pairs to secure the stem−loop. To examine if aminoglycoside antibiotics can bind to TS mRNA, four of the rhodamine-derivatized aminoglycosides (CRP, CRG, CRT, and CRK) (Chart 2) were tested for their binding affinity with the site 1 construct of the TS mRNA by fluorescence anisotropy. The aminoglycosides were incubated with increasing concentration of the site 1 TS mRNA construct, and their corresponding fluorescence anisotropy values were best fitted to afford their respective binding constants (K_ds). All the derivatized aminoglycosides showed comparable binding affinities, with the rhodamine-derivatized paramomycin (CRP) showing the highest binding affinity with a measured dissociation constant of 0.73 μM (Table 1). A representative binding isotherm for CRP is shown in Figure 3. The binding shows saturable behavior and is fit to a 1:1 stoichiometric mode of binding (*11, 32*). In subsequent experiments, the CRP−TS mRNA construct complex was used in the competition assays to measure the binding of various aminoglycosides.

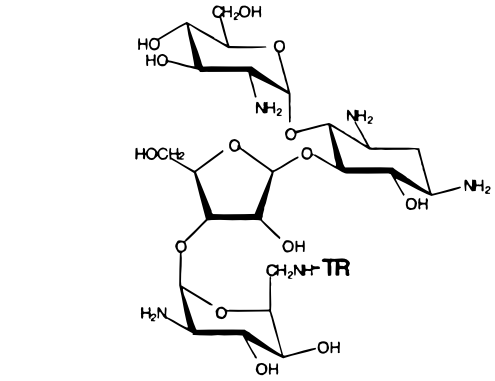
Binding Characteristics of Aminoglycosides with the Site 1 TS mRNA Construct. A series of commercially available aminoglycoside antibiotics, shown in Chart 3, were studied with respect to their abilities to compete with CRP for binding to the TS mRNA construct. In these experiments, a solution of CRP (10 nM) and RNA (400 nM) was used as the assay reagent. Increasing concentration of added aminoglycosides caused a decrease in the initial fluorescence anisotropy values in a saturable manner. The K_d values for the individual aminoglycosides were calculated by curve-fitting to eq 2 (Materials and Methods). Neomycin B was observed to have the highest affinity for the construct among the series of aminoglycoside antibiotics screened and had a dissociation constant of 0.88 μM (Table 1). Paramomycin, kanamycin B, and gentamycin C showed comparable binding constants in the range of 1.87−2.43 μM, whereas streptomycin and hygromycin showed weak or no specific binding to the construct.

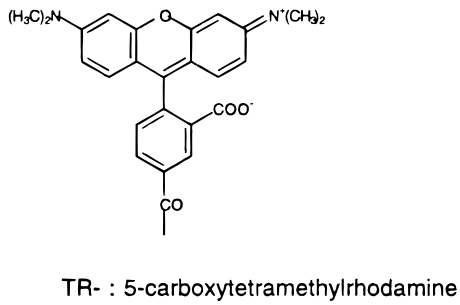
Studies on the Binding Site of Aminoglycoside in the Site 1 TS mRNA by Mutational Analysis. To determine the nature of the aminoglycoside binding-site in the TS mRNA construct, a series of mutational studies were carried out. In the construct **1** used, additional GC base pairs were added to secure the stem structure. These GC pairs added a second internal bubble not found in the native construct (Chart 1). To determine if either of the bubbles was responsible for aminoglycoside binding, a construct was prepared that closed both bubbles (construct **2**). The two bubbles 1 and 2 in the stem region were mutated to afford construct **2** with a complete "closed up" stem region (Chart 1). Binding studies with the four fluorescent aminoglycosides failed to detect any measurable binding by fluorescence anisotropy measurements. This observation indicates that the binding of the aminoglycosides probably takes place at the stem region of the site 1 TS mRNA and not at the hairpin loop of the construct. The next two constructs studied involved the sequential closure of the two bubbles 1 and 2 in the stem region (construct **3** and **4** shown in Chart 1). By fluorescence anisotropy measurements, it was shown that when bubble 2 was "sealed up" through mutagenesis to generate construct **3**, the binding ability of aminoglycosides was abolished. On the other hand, when bubble 1 was sealed up by mutagenesis to afford construct **4**, the binding affinity of the aminoglycosides was not greatly affected as compared to the wt TS mRNA construct **1** (Table 2). These observations show that aminoglycoside binding is due to the native CC bubble in the wt construct and not due to the hairpin loop or the second bubble introduced into the construct.

To confirm that bubble 2 is the probable site of aminoglycoside binding, a "hybrid" RNA construct was created by joining the hairpin loop section of the wt site 1 TS mRNA construct (retaining the CC bubble) with the stem region of the A-site 16S rRNA construct (*15*). Native A-site constructs are only able to bind aminoglycosides when they have a decoding region bubble intact; in its absence, the stem−loop structure is not competent to bind aminoglycosides (*16, 17*). The resulting hybrid prepared here, which contains the CC bubble (construct **5**), is shown in Figure 4, with its secondary structure predicted with the Mfold RNA program of Zuker (*35*). This "hybrid" construct is competent to bind aminoglycosides as revealed through fluorescence anisotropy measurements. The binding affinities for aminoglycosides is only slightly decreased compared to the wt site 1 TS mRNA construct (Table 3).

Determining the Binding Site of Aminoglycoside in Site 1 TS mRNA by Footprinting Studies: (1) RNase CL3 Foot-

Chart 2: Rhodamine-Derivatized Aminoglycoside Tracers Used in the Binding Assay for Site 1 TS mRNA





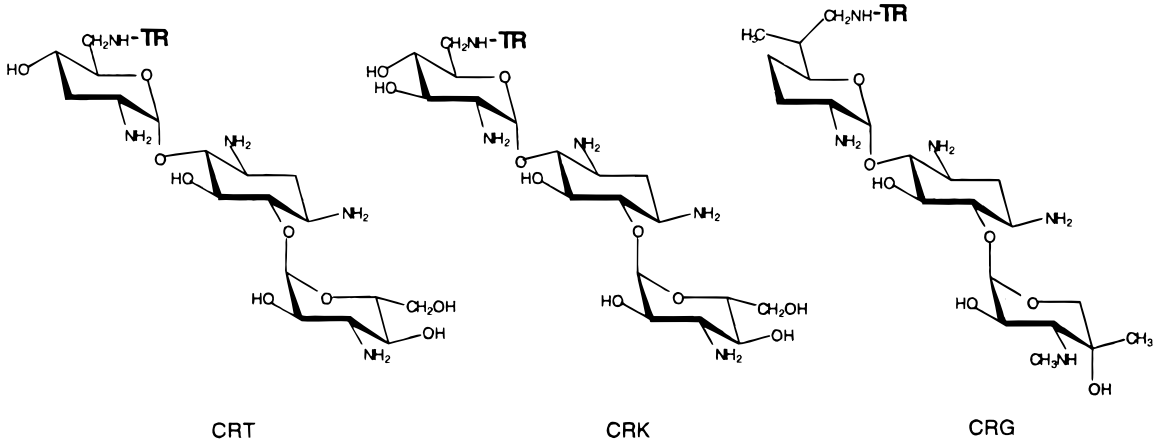


Table 1: Binding Constants of (A) Underivatized Aminoglycosides and (B) Rhodamine-Derivatized Aminoglycosides with Construct **1**

	K_d (μM)
(A) Aminoglycosides	
neomycin B	0.878 ± 0.096
paramomycin	2.321 ± 0.152
gentamycin C	2.028 ± 0.239
tobramycin	2.185 ± 0.278
kanamycin B	2.718 ± 0.488
streptomycin	nb[a]
hygromycin	nb
(B) Derivatized Aminoglycosides	
CRP	0.733 ± 0.041
CRG	0.849 ± 0.050
CRT	0.815 ± 0.063
CRK	0.947 ± 0.087

[a] nb, no binding.

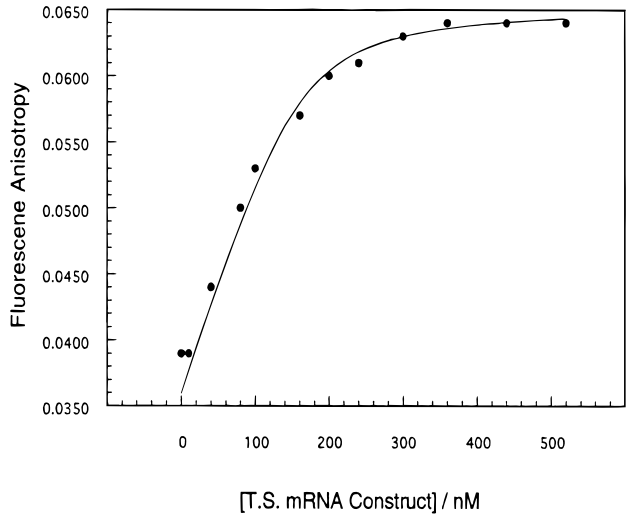


FIGURE 3: Fluorescence anisotropy of CRP (10 nM) as a function of site 1 TS mRNA (construct **1** in Chart 1) concentrations.

printing of Site 1 TS mRNA. To demonstrate that the aminoglycoside binding site of the construct at least partly resides in the CC bubble, RNase CL3 footprinting experiments were performed on the site 1 TS mRNA construct. RNase CL3 is expected to cleave 3′ to cytidines in the single-strand region of the TS mRNA construct (*36*). As shown in Figure 5, RNase CL3 readily cleaves the site 1 TS mRNA at C82−84, C87, C90, C92−93 and G97. Neomycin protected C82, C84, C87, and C90 when added at a concentration of 100 μM. Most of the C residues in the internal bubble except C83 were protected from RNase CL3 cleavage, whereas the C residues in the loop were not protected. This result indicates that two bubbles in site 1 TS mRNA construct may interact with neomycin.

(2) Interaction of the Phosphate Backbone of the Site 1 TS mRNA Construct with Neomycin. To identify possible phosphate contacts with neomycin, an ethylation interference experiment was performed with the neomycin column

Chart 3: Aminoglycosides Used in Screening Assay for Binding to Wild Type and Mutants of Site 1 TS mRNA Construct

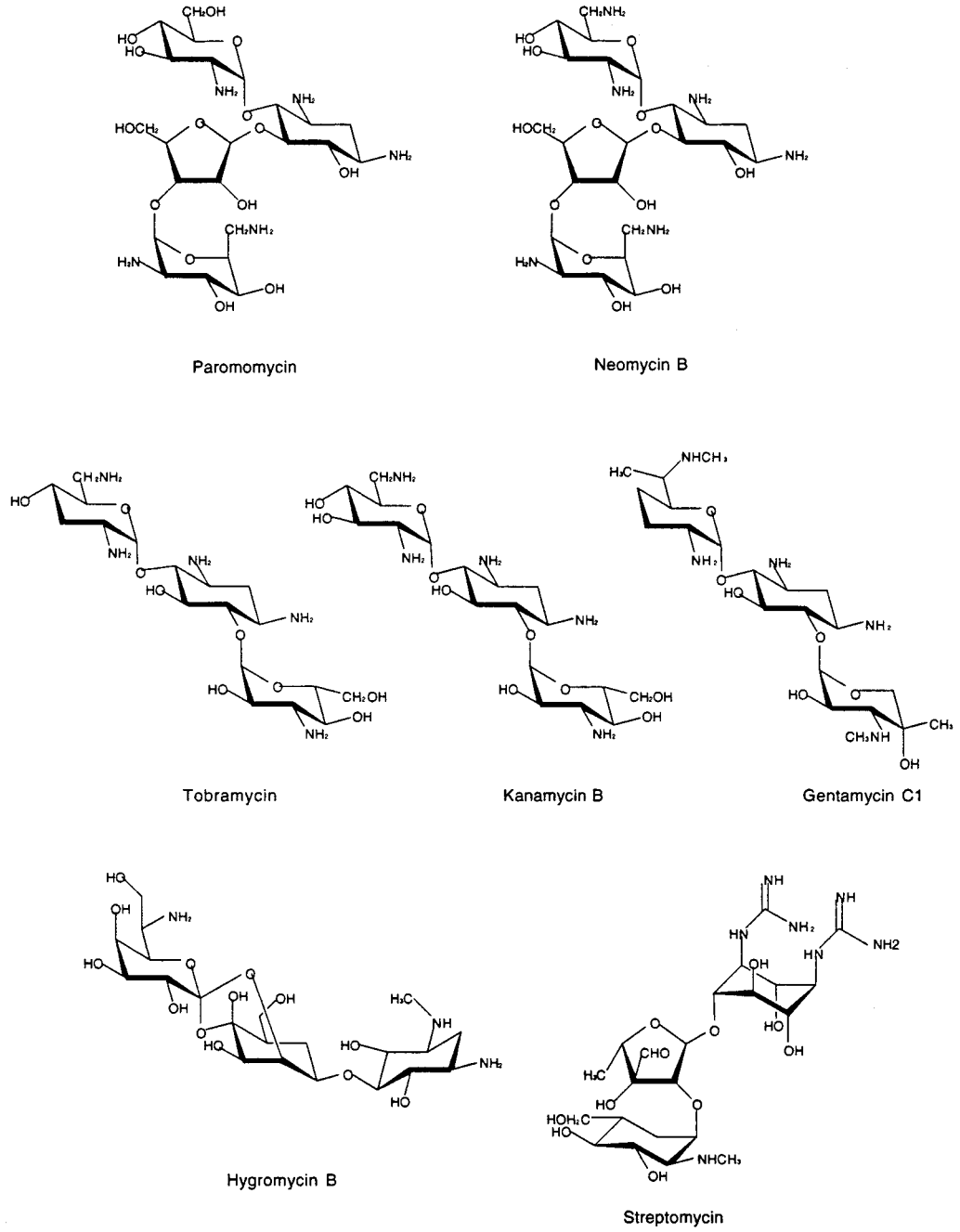


Table 2: Binding Constants of (A) Underivatized Aminoglycosides and (B) Rhodamine-Derivatized Aminoglycosides with Mutant Construct **4** of Site 1 TS mRNA

	K_d (μM)
(A) Aminoglycosides	
neomycin B	0.977 ± 0.054
paramomycin	1.863 ± 0.113
gentamycin C	2.544 ± 0.182
tobramycin	2.292 ± 0.280
kanamycin B	2.428 ± 0.308
(B) Derivatized Aminoglycosides	
CRP	0.714 ± 0.066
CRG	0.919 ± 0.080
CRT	0.877 ± 0.079
CRK	1.107 ± 0.091

described in Materials and Methods. The phosphate groups in site 1 TS mRNA were ethylated with ethyl nitrosourea (ENU), and the modified RNAs were bound to the column, eluted with an increasing salt gradient, cleaved with aniline, and run on gels.

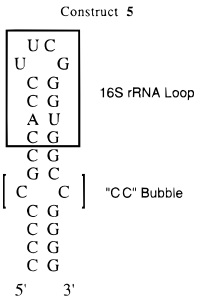


FIGURE 4: Hybrid RNA construct **5**, in which the stem region of the A-site 16S rRNA analogue (*14*) was fused with the hairpin loop of the site 1 TS mRNA construct.

Table 3: Binding Constants of (A) Underivatized Aminoglycosides and (B) Rhodamine-Derivatized Aminoglycosides with Mutant Construct **5** of Site 1 TS mRNA

	K_d (μM)
(A) Aminoglycosides	
neomycin B	1.448 ± 0.101
paramomycin	2.241 ± 0.210
gentamycin C	2.833 ± 0.241
tobramycin	3.328 ± 0.293
kanamycin B	3.492 ± 0.322
(B) Derivatized Aminoglycosides	
CRP	1.117 ± 0.094
CRG	1.245 ± 0.097
CRT	1.309 ± 0.128
CRK	1.482 ± 0.139

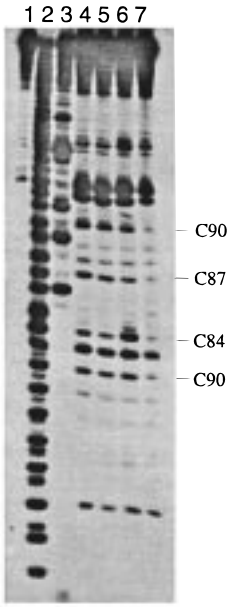


FIGURE 5: RNase CL3 footprint of neomycin on site 1 TS mRNA. Autoradiogram of a 15% denaturing polyacrylamide gel showing Ce(III) ion-mediated footprinting of 5′-^{32}P-labeled TS RNA. (Lane 1) Intact TS RNA; (lane 2) alkaline hydrolysis; (lane 3) G-specific RNase T1 sequencing reaction; (lane 4) RNase CL3 cleavage control reaction; (lanes 5−7) RNase CL3 cleavage reaction in the presence of 1, 10, and 100 μM neomycin.

As shown in Figure 6, modification of C81−C88 phosphates, located at the 5′ side of the bubble, interfered with site 1 TS mRNA binding to the neomycin column. Neomycin appears to asymmetrically interact with the phosphate backbone of site 1 TS mRNA. Together with RNase CL3 footprinting result, the current findings suggest that the bubbles of site 1 TS mRNA are at least part of neomycin binding site (Chart 4). Given the results from the mutational studies, the bubble structure 1 is essential for aminoglycoside binding.

DISCUSSION

Aminoglycosides operate as antibacterial agents by binding to the prokaryotic A-site decoding region of the 16S rRNA (*1*−*4*). The binding of aminoglycosides to this site is not of a very high affinity kind, but rather occurs in the low micromolar range (*14*, *32*). The fact that structurally diverse aminoglycosides bind to this site suggests that not a great deal of specificity is involved in the binding. The aminoglycoside binding domain consists of an internal bubble structure

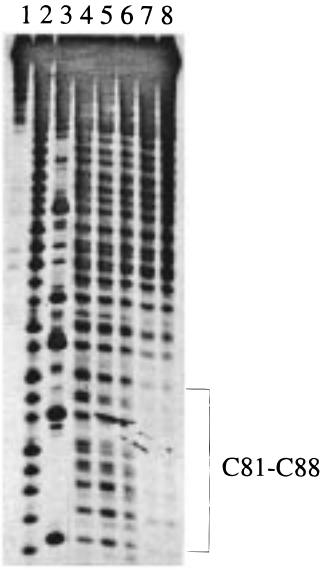
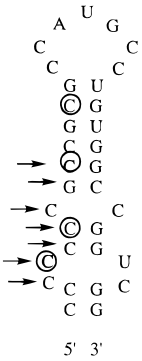


FIGURE 6: ENU modification interference of neomycin binding to site 1 TS mRNA. Autoradiogram of a 15% denaturing polyacrylamide gel showing ethyl nitrosourea (ENU) modification interference of 5′-^{32}P-labeled TS RNA. (Lane 1) Intact TS RNA; (lane 2) alkaline hydrolysis; (lane 3) G-specific RNase T1 sequencing reaction; (lane 4) ENU control reaction; (lanes 5−8) eluted RNAs with an increasing NaCl gradient (0.1−1 M).

Chart 4: Summary of RNase CL3 Footprinting and ENU Interference Results on Site 1 TS mRNA Construct



(*14*), and NMR studies have afforded some information on the nature of the interactions which mediate aminoglycoside−RNA recognition in this instance (*15*). Other RNA molecules have also been discovered that bind to aminoglycosides. High-affinity (nanomolar) RNA aptamers selected to bind to the aminoglycoside tobramycin have binding sites localized in internal bulge regions (*11*, *31*, *37*), and the HIV RRE region binds aminoglycosides at internal bulge structures (*12*, *13*). To further explore the kinds of local RNA structures that might interact with aminoglycosides, we have studied the putative binding of aminoglycosides to a TS mRNA construct that contains a CC bubble in a stem−loop region. The loop region contains the initiation codon AUG for TS translation (*30*).

When the RNA construct **1** (modeled after the site 1 TS mRNA) was prepared, it was found to specifically and stoichiometrically bind aminoglycosides with affinities quite similar to that found in the case of the A-site prokaryotic rRNA decoding region (*32*). Moreover, the rank order of binding also appeared similar; neomycin bound with the highest affinity, and neither streptomycin nor hygromycin bound (*32*). The functional binding of aminoglycoside to the

TS construct **1** importantly depends on the CC bulge region. Mutation of one of the Cs to a G abolishes specific aminoglycoside binding, even though a second bulge still is present in the construct. Interestingly, the second bulge region allows for only weak, nonspecific, aminoglycoside binding. It would be interesting to determine the structural differences between the two internal bulge sites and how this translates into the ability to specifically bind aminoglycosides. The loop region of the construct also appears not to be an important contributor to aminoglycoside binding because the construct **2** with both bulges closed up does not bind aminoglycosides. In general, it has been found that when mutations are introduced to the internal bulges or bubbles of simple duplex stem−loop RNA molecules, e.g, the A-site 16S rRNA decoding region construct, the RNA construct is rendered incompetent to specifically bind aminoglycosides (*16*, *17*).

Interestingly, the CC bulge aminoglycoside binding domain of the TS construct was able to confer aminoglycoside binding abilities on the stem−loop domain of the A-site decoding region. This result suggests that the binding of aminoglycosides to the CC bubble domain is largely context-independent. The result is also consistent with the notion that the binding of aminoglycosides to RNA molecules can occur through local structures and that these structures are often of the internal bubble or bulge type. The binding of aminoglycosides to internal structured regions of RNA is, however, hardly nonspecific in its nature. For example, often internal bubble structures are incapable of binding aminoglycosides. Point mutations in the A-site decoding region result in an RNA molecule unable to bind aminoglycosides (*16*, *17*), and the P-site decoding region, an internal bubble structure (*14*), does not bind aminoglycosides (*18*). Finally, the second bubble in the TS mRNA construct described here is unable to bind aminoglycosides with appreciable affinity. On the basis of the footprinting experiments described here, however, it is likely that the two adjacent bubbles of the TS construct may interact to some extent. Binding of aminoglycosides to bubble 1 probably alters the conformation of the nts in the second bubble, and this is reflected in the results showing changed reactivity in the latter, even though aminoglycosides are not directly bound.

The experiments described here show that aminoglycosides can bind specifically and with moderate affinity to an RNA construct modeled after site 1 mRNA. This 5′-UTR site is of substantial pharmacological interest in that it appears to be a locus of interaction between apo-TS and mRNA (*22*, *23*). In its non-substrate- or inhibitor-bound form, apo-TS binds to mRNA and prevents translation of message, presumably by interfering with translation factors that bind to the same, or overlapping, sites on the mRNA (*22*). When TS is bound to substrates or inhibitors, such as the anticancer drug 5-fluorouridylate (*38*−*40*), translational repression is lost and overproduction of TS can ensue, resulting in the development of resistance to the drug (*38*, *39*). Thus, selective and potent small molecules directed against the unique site 1 TS mRNA site could be of potential interest as anticancer drugs by inhibiting the translation of TS message. The currently available aminoglycosides are unlikely to be potent or selective enough to be serious candidates in this regard. However, the 1,3(2)-amino alcohol pharmacophore of aminoglycosides (*7*, *8*) may serve as a beginning point for the design of molecules that might be of interest as candidate drug entities to interfere with TS synthesis.

The present study establishes an additional naturally occurring RNA receptor for aminoglycosides. Other RNA targets for aminoglycoside antibiotics (Chart 3) include the previously mentioned prokaryotic 16S rRNA A-site decoding region (*14*, *15*), the HIV-1 RRE transcriptional activator region (*12*, *13*), class 1 introns (*42*, *43*), and hammerhead ribozymes (*44*). A great challenge in the design of anti-RNA drugs will be to begin to differentiate among these various RNA targets with specific small molecules.

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